CALCIFICATION XIV.

INVESTIGATION OF THE ROLE OF CHONDROITIN SULFATE IN THE CALCIFYING MECHANISM

by

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The purpose of these investigations was to evaluate the possible role of chondroitin sulfate (or some related mucopolysaccharide) in the mechanism of calcification. There are many studies that indicate the possible role of chondroitin sulfate as an integral part of the minimum mechanism required for mineralization. Sulfate-containing mucopolysaccharides appear whenever calcification takes place; namely, in the dentin and the enamel (1), in the bone (2), and in abnormal calcification of the arteries (3). Rubin and Howard (4) indicated the presence of metachromatic staining in all types of pathological calcification studied, i.e., calcinosis universalis, calcified bursitis, and renal stones of the calcium, phosphate, carbonate type. Significantly, they found no indication of mucopolysaccharides in calcium oxalate and ammonium urate stones. Miller, Waldman and McLean (5), based on the inhibition of calcification in vitro of rachitic cartilage by dyes such as toluidine blue and other basic and acid dyes state, "Our experiments suggest that chondroitin sulfate may be essential for the calcification of hypertrophic rachitic cartilage." Einbinder and Schubert (6) have shown that collagen, under certain conditions, will react with chondroitin sulfate so vigorously that sulfate will replace absorbed dyes from the collagen. Hyaluronic acid and other mucopolysaccharides will not do this. Since collagen is the key protein of bone and dentin these experiments again suggest that this ability of chondroitin sulfate may be one of the properties of the protein which in combination is responsible for initiating calcification.

Sobel and his colleagues (7-9), based on their studies of the reversible inactivation of in vitro calcification, suggested that "combination with calcium of some constituent of the ossifying matrix is an essential preliminary step in the mineralization process." They further presented evidence in the literature to indicate that chondroitin sulfate may be the target of the inactivating (Be^{++}, Mn^{++}, Cu^{++}, Mg^{++}, Ba^{++}, Na^{+}, and Sr^{++}) and the activating (Ca^{++}) ions. They suggested that perhaps the activating role of calcium ions consists of the polymerization of some mucopolysaccharide, probably chondroitin sulfate.

According to Partridge (10) calcium is particularly effective in causing a shortening in length of collagen fibers, with a subsequent release of chondroitin sulfate. Calcium ion increases the viscosity of at least some mucopolysaccharides, thus indicating polymerization (10). This polymerization, at least for hyaluronate by means of calcium ion, was related to the rate of prothrombin conversion to thrombin (11).

Further investigations were undertaken to clarify the role of the metachromatic staining substance (almost certainly chondroitin sulfate) (12) in the calcifying mechanism present in the preosseous cartilage. Inactivation with protamine and toluidine blue (compounds which combine avidly with mucopolysaccharides) is a function of the inactivator to calcium ratio, as shown in Table 1. Table 2 shows that when toluidine blue is present in the calcifying medium the degree of inactivation is a function of the dye concentration. From these studies one might postulate that chondroitin sulfate is an integral part of the "local factor" responsible for calcification.

Next we investigated in parallel experiments the degree of metachromasia and the calcifiability of rachitic bone sections. In these studies sections, after appropriate treatment, were stained with dye and observed under 32 to 96 magnifications. In this way one avoids chemical changes due to the usual histological procedures.

Since calcium chloride enhances the degree of calcifiability and serves the unique role of reactivating the calcifying mechanism (7,9), metachromasia following this treatment was compared to controls. Here again, a parallel was observed between the degree of calcifiability and metachromasia. This was true for heated sections as well (See Table 3). This parallelism between metachromasia and calcifiability is again observed following treatment with xylene. Before calcium chloride shaking the metachromasia is low and calcifiability is absent under the conditions of our experiment. However, after calcium chloride treatment, there is intensification of meta-

chromasia as well as a manifestation of calcifiability.

No parallel behavior was observed between calcifiability and metachromasia after treatment with formaldehyde, methyl alcohol, and 95% phenol (95 g. phenol plus 5 g. water). Here metachromasia was exceptionally intense, but calcifiability was not evident. From these studies it was tentatively postulated that since methyl alcohol, phenol and formaldehyde are protein denaturants, the complex of the chondroitin sulfate - protein bond may be altered and the intact combination is necessary for the calcifying mechanism.

The next experiments were based on the observations that the maximum degree of metachromasia took place at the calcium ion concentration of about 15 meq/L in studying the influence on in vitro calcification of toluidine blue to calcium ratios, presented in Table 1. Moreover, they were based on the postulate made previously by Sobel and Hanok (9) that the activating role of calcium ion consists of the polymerization or some other change in the configuration of chondroitin sulfate attached to a protein molecule. This concept is presented schematically in Figure 1, based on the results obtained in the experiments on reversible inactivation. In Figure 1, the symbols for "inactive" and "active" refer to the "local factor" responsible for the mechanism of calcification. The changes in shape are not meant to imply the actual shape of the molecule(s), but simply to indicate changes per se. For this reason studies were undertaken to determine the degree of metachromasia with constant amounts of toluidine blue in the presence of varying amounts of calcium ions. Typical results are given in Figure 2. The results shown in the figure were repeated, with minor variations, five times. Calcium ion up to 15 meq/L enhances the degree of metachromasia. Above this concentration of calcium ion, the degree of metachromasia slowly diminishes. This behavior of chondroitin sulfate in the rachitic bone section is in marked contrast to that observed when metachromasia was produced with chondroitin sulfate prepared from bone cartilage or nasal septum cartilage. Here, calcium ion in

concentrations as low as 0.2 meq/L prevented or destroyed metachromasia. For this reason it was proposed that chondroitin sulfate in the rachitic bone is in combination with some other molecule, probably collagen, and that if chondroitin sulfate is a mediator of calcification it is due to this complex. Such a complex can be visualized as analogous to an enzyme, with a prosthetic group attached to a protein. The two together are necessary for enzyme action. By analogous reasoning, one can visualize chondroitin sulfate attached to a protein, and when the complex is in a certain state of configuration it becomes a catalyst or activator of crystallization.

From the results obtained the process visualized is that of calcium plus toluidine blue competing for the chondroitin sulfate complex. Calcium ion up to a certain concentration causes a rearranged chondroibin sulfate complex, so that it becomes more active metachromatically, probably a state of polymerization. After reaching this new configuration, with further increased concentrations of calcium ions, competitive behavior becomes evident by decreased metachromasia, since the calcium binding is no longer compensated by further intensification of metachromatically active charges in the molecule.

That this is a combination of chondroitin sulfate and collagen is indicated in preliminary studies. Collagen prepared from Achilles tendon was condensed with chondroitin sulfate according to the Einbinder-Schubert method (6). The resultant product was treated with toluidine blue in the presence of calcium ions, as in the case of rachitic bone sections. The results are shown also in Figure 2, and denote an increased degree of metachromasia up to 15 meq/L, after which a gradual decline took place. The control collagen showed only the faintest trace of blue under the conditions of our experiment (one hour) without metachromasia. The chondroitin sulfate treated with calcium ions exhibited no metachromasia, but did exhibit metachromasia in the absence of calcium. This complex shaken with 0.25 molar calcium chloride followed by 0.32 molar phosphate, gave a silver stain similar to that found

in in vitro calcification. Collagen alone did not exhibit this phenomenon. These studies seem to imply that the metachromatically active substance is probably chondroitin sulfate attached to collagen fiber in a critical configuration. This configuration may represent a critical portion of the "local factor" in the mechanism of calcification.

The next experiments were undertaken to determine the changes in metachromasia with respect to calcium ion concentration in bone sections where, after treatment, calcifiability was destroyed but metachromatic activity was still present (Table 3). If there is a change in the combination between chondroitin sulfate and the collagen, a clue to the nature of such changes may be obtained. For example, if chondroitin sulfate is completely free, calcium ions should interfere with metachromasia. Bones were examined after treatment (a) for six hours in basal salt solution, (b) for one hour with 38% formaldehyde, and (c) for one hour with 95% phenol. Maximum metachromasia was obtained in the absence of calcium ion in the basal salt and formaldehyde treated sections, and with only one meq/L in the phenol treated sections. This maximum began to decline at 20 meq/L calcium ion, and metachromasia completely disappeared in the case of phenol and basal salt solutions at 50 meq/L calcium ion. For formaldehyde the metachromasia disappeared between 50 and 100 meq/L calcium ion. These studies may indicate that a characteristic of bone cartilage with intact calcifying mechanism is its ability to respond with an increasing degree of metachromasia under the influence of calcium ion. When the calcifying mechanism is destroyed with survival of the metachromatically active substance a new configuration developed. Here calcium ion no longer enhanced the degree of metachromasia, and interfered with metachromasia at lower concentrations of calcium ion. One possible explanation is that some of the chondroitin sulfate is freed of the protein bond.

In future studies, chondroitin sulfate prepared from various sources will be condensed with collagen prepared from various tissues by several methods. These

collagen - chondroitin sulfate complexes will be examined by methods analogous to *in vitro* calcification to determine which of these may be responsible for the formation of bone-like minerals. If these experiments are successful an attempt will be made to condense keratin (the key protein of enamel) with chondroitin sulfate to determine whether these complexes may initiate production of mineral similar in structure to that found in enamel. With all the above compounds the influence of calcium ion on the degree of metachromasia will be investigated, and if possible, used as a guide to mineralization.

EXPERIMENTAL

The solutions employed were a stock solution of toluidine blue "O" [1] (T.B.O) containing 10 mg./100 ml. (1.58×10^{-4} M), which was diluted as required (usually a 1:4 dilution was used); a stock solution of $CaCl_2$ (prepared from the carbonate) containing 500 meq/L of Ca^{++}, which was diluted as required; a 3% $AgNO_3$ solution for use in the "line test" to determine the degree of calcification and solutions of chondroitin sulfate prepared from rachitic bone cartilage or from beef nasal septum cartilage[2]. All solutions were prepared with ion exchange water which was redistilled in an all glass (Pyrex) distillation apparatus.

The organic reagents used were: 95% phenol (95 g. recrystallized phenol in 5 g. of distilled water), 38% formalin, absolute methanol, and 95% ethanol.

The bones used were the excised tibiae of rachitic Wistar strain albino rats. The animals were maintained on a rachitogenic diet for three to four weeks, when rickets was severe, and were sacrificed by chloroforming. The diet consisted of 76% degerminated yellow corn meal, 20% wheat gluten, 3% $CaCO_3$, and 1% NaCl. Ion exchange water was supplied ad libitum. The excised tibiae were freed from adhering soft tissue and the upper ends were sliced longitudinally into sections less than 1 mm. in thickness.

To insure homogeneous exposure to the calcifying and toluidine blue "O" solutions, the sliced sections were suspended from glass hooks (made from melting point tubes) inserted in paraffined cork stoppers (13). Shaking of sections was always done in a mechanical shaker.

The collagen[3] used was prepared from the Achilles tendon of steer by removing the skin from the top and cutting the tendon into small pieces. The pieces were washed overnight with cold running tap water, covered with half saturated calcium hydroxide solution and stirred mechanically for one hour at room temperature. At

this time the stirrer was removed and the solution was decanted. Fresh calcium hydroxide solution was added and the stirring was resumed for another hour. This process was repeated five times. The calcium hydroxide extracted protein was rinsed with running tap water for four hours, followed by several rinses with distilled water. The final residue was air dried and consisted mainly of collagen with small amounts of elastin.

The collagen - chondroitin sulfate complex was prepared according to the method of Einbinder and Schubert (6). The control collagen was devoid of chondroitin sulfate.

The method of evaluating the degree of calcification in vitro was essentially that described by Sobel (14) and Sobel and Hanok (9).

The degree of metachromatic staining (metachromasia) in the provisional zone of the epiphyseal cartilage was evaluated with a Leitz wide field stereo binocular microscope. A magnification of 32X was usually used. The degree of metachromasia was graded as follows:

0 = no purple (metachromatic) stain.

± = broken thin purple line extending latitudinally along the epiphyseal cartilage plate.

+ = continuous thin purple line extending along the epiphyseal plate.

++ = continuous purple line extending longitudinally half way up the epiphyseal plate.

+++ = continuous purple line extending longitudinally three quarters of the way up the epiphyseal plate.

++++ = complete purple stained epiphyseal cartilage.

Prior to any treatment, sections from each animal were placed in glass, color reaction, depression plates and covered with 3% silver nitrate solution. They were

then exposed to light from a tungsten filament lamp for 5 minutes to determine the presence of rickets. If a silver stain was obtained (indicating healing of rickets) the animal was discarded. Similar glass depression plates were used to examine the degree of metachromasia. This was done by placing the section on the plate, covering it with distilled water, and examining by reflected light. Quite often we observed sections with such intense metachromasia that it appeared bronze-like rather than purple. However, if such a section was sliced with a razor blade or with a microtome and examined by transmitted light, a deep purple-red stain was observed.

After staining with the dye, some sections were soaked in 95% alcohol prior to microscopic observation. This served to remove flase metachromasia as recommended by Montagna, et al.(15).

Usually after one hour of soaking in the toluidine blue "O" solution, the metachromatic staining appeared as a pericellular aggregation about the cartilage cells. This pericellular aggregation was not so evident in the sections that were treated with formalin, phenol and methanol. These observations confirm those made by Follis (16).

SUMMARY

1. Studies were undertaken to evaluate the possible role of chondroitin sulfate or some related mucopolysaccharide in the mechanism of calcification.

2. The results obtained indicate that a complex of chondroitin sulfate in a highly specialized configuration may be an integral component of the minimum mechanism required for calcification.

3. That chondroitin sulfate may be involved in the calcifying mechanism of rachitic bone is suggested from studies with toluidine blue and protamine, which inactivate this mechanism. This inactivation is a function of the inactivator to calcium ratio.

4. In attempting to relate the "local factor" to the state of polymerization of chondroitin sulfate, the influence of calcium ions on metachromatic staining was investigated. With a constant amount of toluidine blue the degree of metachromasia increases with calcium ion concentration in solution, up to a maximum of about 15 meq/L. Above this concentration of calcium ions there is a gradual decrease in metachromasia. Prior shaking with calcium chloride increases intensity of metachromatic staining in the ossifying matrix. In contrast to this, when chondroitin sulfate is extracted from bone, calcium ion competitively interferes with metachromatic staining. However, the metachromasia obtained with chondroitin sulfate combined with collagen responds to calcium ion in the same way as rachitic bone cartilage.

5. This property of calcium ions increasing the degree of metachromasia seems to be typical of calcifying cartilage in the studies carried out to date. The appearance of metachromatic staining in bone cartilage did not correlate in all cases with calcifiability. However, when calcifiability was destroyed by various agents, metachromasia was not enhanced by calcium ions.

FOOTNOTES

[1] Obtained from Hartmann - Leddon Co., C.I. No. 925, Philadelphia, Pennsylvania.

[2] Kindly supplied by S. Roseman, University of Chicago, Chicago, Illinois, and E. Jorpes, Karolinska Institute, Stockholm, Sweden.

[3] Prepared by S. Natelson, Rockford Memorial Hospital, Rockford, Illinois.

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TABLE 1

INFLUENCE OF TOLUIDINE BLUE "O" OR PROTAMINE : Ca RATIOS ON SUBSEQUENT CALCIFICATION IN VITRO

INACTIVATION		
Toluidine Blue "O" Solution	$CaCl_2$ Solution	Degree of Calcification
µM/L	meq/L	mean
39	0.0	0
39	1.0	trace
39	5.0	trace
39	10.0	1(+)
39	30.0	1(+)
39	50.0	1(+)
39	70.0	1(++)
39	100.0	1(+++)
39	150.0	1(+++)
39	300.0	2(++)
39	100.0	0.5(++)
1	139.0	1.3(++++)
0.6	144.0	1.7(++++)
0	0.0	0
0	150.0	2(++++)
Protamine Solution	$CaCl_2$ Solution	Degree of Calcification
per cent	meq/L	mean
0.4	5.0	0
0.4	150.0	1(++++)*

*Surface covered, calcification was below the surface, became evident on cleaning of the sections.

Degree of calcification of untreated controls (mean) = 2(++++).

Following preliminary shaking in toluidine blue "O" - $CaCl_2$ solutions for one hour, and protamine - $CaCl_2$ solutions for two hours, sections were placed in calcifying solution for 18 hours at 37°, pH 7.3± 0.07. Ca = 10 mg.%, P = 5 mg.%.

TABLE 2

CALCIFICATION IN VITRO IN THE PRESENCE OF TOLUIDINE BLUE "O"

T.B.O*	Degree of Calcification	Degree of Metachromasia
uM/L	mean	mean
15.8	0	doubtful
5.3	1.0(++++)	doubtful
2.2	1.2(++++)	0
1.7	1.0(++++)	0
0	2.3(++++)	—

*T.B.O = Toluidine blue "O".

T.B.O was placed in the calcifying solution, Ca = 10 mg.%, P = 5 mg.%, at 37° C. for 18 hours, pH 7.3±0.07.

TABLE 3

METACHROMASIA AND CALCIFICATION BY VARIOUS TREATMENTS

TREATMENT	Before Shaking With $CaCl_2$		After 1 Hr. Shaking With 150 meq/L $CaCl_2$	
	Meta-chromasia	Calcification	Meta-chromasia	Calcification
Shaken 2 hr. in basal salt sol'n at 37° C.	+++	1(++++)		
Shaken 6 hr. in basal salt sol'n at 37° C.	++	0		
Shaken 2 hr. in basal salt sol'n containing 5 meq/L Ca^{++}, 37° C.	++++	2(++++)		
Shaken 6 hr. in basal salt sol'n containing 5 meq/L Ca^{++}, 37° C.	++++	2(++++)		
Shaken 1 hr. in distilled water at room temperature.	±	0	+++	2.5(++++)
Heated 30 min. in distilled water at 97°C.	++	0	+++	2(++++)*
Shaken 1 hr. in xylene	±	0	++±	2.5(++++)*
Shaken 1 hr. in ethyl alcohol, 95%	++	1(+)		2(+++)
Shaken 1 hr. in ethyl alcohol, 95% + solid $CaCO_3$		2(+++)		4(++++)
Shaken in 38% formalin	++++†	0	++++†	0
Shaken 1 hr. in methyl alcohol, absolute	+++†	0	+++†	0
Shaken 1 hr. in 95% phenol (95 g. phenol + 5 g. water)	+	0	+++†	0

* See Sobel, A.E., and Hanok, A., Table VII (9)

† Metachromasia was extremely intense and appeared as a velvety, bronze colored structure. Microtomed sections, viewed by transmitted light, appeared magenta colored.

REVERSIBLE INACTIVATION OF CALCIFICATION IN VITRO (TENTATIVE MECHANISM)

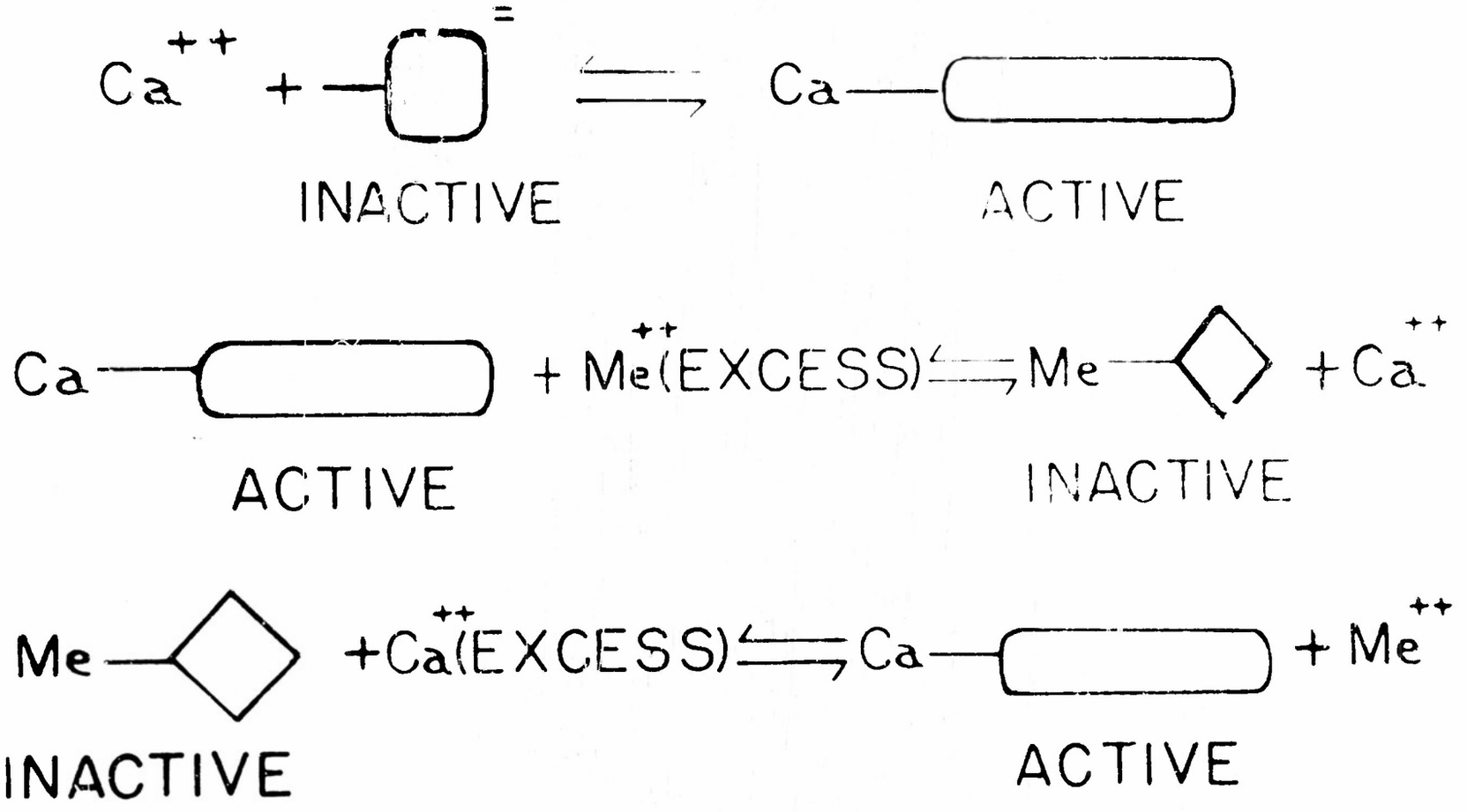

Fig. 1

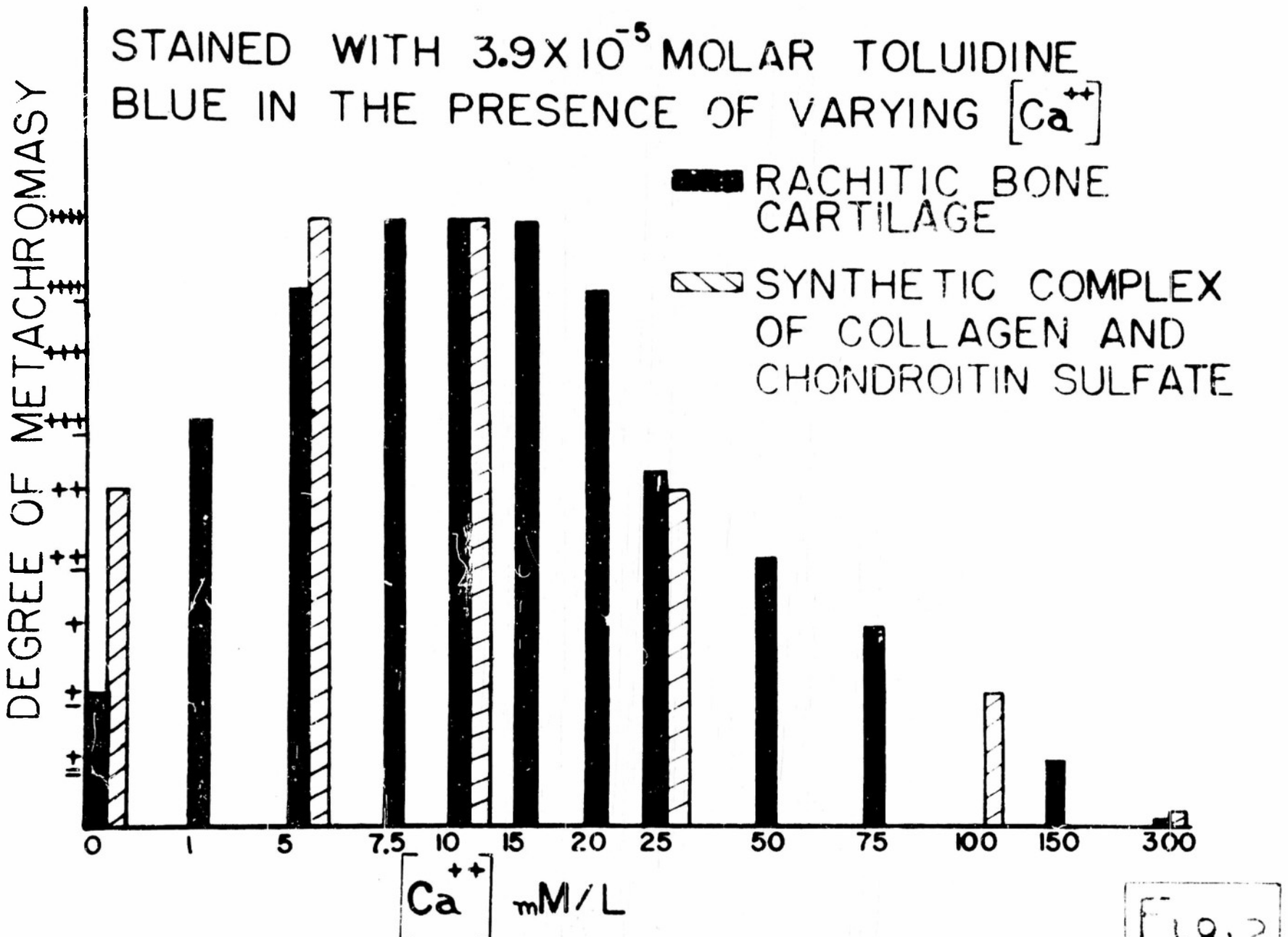
STAINED WITH 3.9×10^{-5} MOLAR TOLUIDINE BLUE IN THE PRESENCE OF VARYING $[Ca^{++}]$
RACHITIC BONE CARTILAGE
SYNTHETIC COMPLEX OF COLLAGEN AND CHONDROITIN SULFATE
DEGREE OF METACHROMASY
0
1
5
7.5
10
15
20
25
50
75
100
150
300
$[Ca^{++}]$ mM/L

Fig. 2